PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/30, 9/16

A1

(11) International Publication Number: **WO 99/24061**

(43) International Publication Date: 20 May 1999 (20.05.99)

(21) International Application Number: PCT/US98/23627

(22) International Filing Date: 6 November 1998 (06.11.98)

(30) Priority Data:

60/064,891	7 November 1997 (07.11.97)	US
60/096,066	11 August 1998 (11.08.98)	US

(71) Applicant: CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).

(72) Inventors: SHIRLEY, Bret; Chiron Corporation, 4560 Horton Street, R–440, Emeryville, CA 94608 (US). HORA, Maninder; Chiron Corporation, 4560 Horton Street, R–440, Emeryville, CA 94608 (US). O'HAGAN, Derek; Chiron Corporation, 4560 Horton Street, R–440, Emeryville, CA 94608 (US). SINGH, Manmohan; Chiron Corporation, 4560 Horton Street, R–440, Emeryville, CA 94608 (US).

(74) Agents: HARBIN, Alisa, A.; Chiron Corporation, Intellectual Property – R 440, P.O. Box 8097, Emeryville, CA 94662–8097 (US) et al.

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR PRODUCING IGF–1 SUSTAINED–RELEASE FORMULATIONS

(57) Abstract

Methods for preparing biodegradable microparticles are provided. Also provided are microparticles prepared by the method which include IGF–1 entrapped therein. The microparticles allow for controlled release of IGF–1 and other polypeptides over prolonged periods of time.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

METHOD FOR PRODUCING IGF-1 SUSTAINED-RELEASE FORMULATIONS

Technical Field

The present invention relates generally to pharmaceutical compositions. In particular, the invention relates to methods for preparing biodegradable microparticles, as well as compositions comprising biodegradable microparticles including entrapped polypeptides, such as IGF-1, for sustained-release.

Background of the Invention

Insulin-like growth factor-I (IGF-1) belongs to a family of polypeptides known as somatomedins. IGF-1 is structurally and functionally similar to, but antigenically distinct from, insulin. In this regard, IGF-1 is a single-chain polypeptide with three intrachain disulfide bridges and four domains known as the A, B, C and D domains, respectively. The A and B domains are connected by the C domain, and are homologous to the corresponding domains of proinsulin. The D domain, a carboxy terminal extension, is present in IGF-1 but is absent from proinsulin. IGF-1 has 70 amino acid residues and a molecular mass of approximately 7.5 kDa. Rinderknecht, *J. Biol. Chem.* (1978) 253:2769; and Rinderknecht, *FEBS Lett.* (1978) 89:283. For a review of IGF, see Humbel, *Eur. J. Biochem.* (1990) 190:445-462.

IGF-1 has been reported to stimulate growth and division of a variety of cell types, particularly during development. See, e.g., EP 560,723 A and 436,469 B. IGF-1 has also been shown to be useful for

the treatment of osteoporosis. See, for example, U.S. Patent No. 5,374,620. Thus, processes such as skeletal growth and cell replication are affected by IGF-1 levels. Furthermore, IGF-1 has been reported to be useful in the treatment of pancreatic disorders (WO 93/25226), renal diseases (U.S. Patent No. 5,106,832) and cardiac disorders (U.S. Patent No. 5,434,134).

Due to the widely varied clinical applications for IGF-1, compositions with desirable characteristics are in great demand and several IGF-1 formulations have been made. See, e.g., U.S. Patent Nos. 5,126,324, 5,374,620 and 5,681,814. These compositions are typically formulated as liquid injectables for parenteral delivery. However, such compositions often require frequent injections which are inconvenient, uncomfortable and subject to poor patient compliance. Furthermore, several disorders for which treatment with IGF-1 is indicated require high doses of IGF-1 not achievable by conventional modes of delivery. Thus, there is a need for IGF-1 compositions which allow for controlled, sustained-delivery of adequate doses of IGF-1.

Particulate carriers have been used in order to achieve controlled, parenteral delivery of therapeutic compounds. Such carriers are designed to maintain the active agent in the delivery system for an extended period of time. Examples of particulate carriers include those derived from polymethyl methacrylate polymers, as well as microparticles derived from poly(lactides) (see, e.g., U.S. Patent No. 3,773,919) and poly(lactide-co-glycolides), known as PLG (see, e.g., U.S. Patent No. 4,767,628). Polymethyl methacrylate polymers are nondegradable while PLG particles biodegrade by random nonenzymatic hydrolysis of ester bonds to lactic and glycolic acids which are excreted along normal metabolic pathways.

Slow-release formulations containing various polypeptide growth factors have been described. For example, International Publication No. WO 94/12158 describes growth hormone controlled-release systems formed by spraying a polymer and dry protein into a freezing solution of liquid nitrogen to form polymeric microspheres. U.S. Patent No. 5,134,122 describes methods of forming microparticles that include salts of peptides such as LHRH. International Publication No. WO 96/37216 describes IGF-1 formulations comprising IGF-1 and hydrophobic polymers. Lam et al., *Am. Assn. Pharm. Sci.* Western Regional Meeting (April 24-25, 1997) Abstract F-21 and Tada et al., *Proc. Intl. Symp. Control. Rel. Bioact. Mater.* (1997) 24:889-890, describe IGF-1 PLG microspheres formed by a spray freeze-drying technique and European Publication No. EP 442,671 A2 describes microcapsules containing various polypeptides.

However, the controlled release of adequate amounts of IGF-1 and other proteins over a defined period remains difficult to achieve. Thus, there is a continued need for IGF-1 sustained-release formulations, as well as methods of preparing microparticle compositions that provide for the continuous release of polypeptides.

Disclosure of the Invention

The present invention is based on the surprising discovery that the use of biodegradable microparticles, such as those derived from a poly(α-hydroxy acid), and including IGF-1 entrapped in the form of a highly concentrated viscous "syrup", provide for continuous delivery of IGF-1 for extended periods of time. This syrup has an IGF-I concentration of at least about 250 mg/ml, a density of about 1.0 g/ml to about 1.2 g/ml, and a viscosity of about 13,000

centipoise (cps) to about 19,000 cps, as measured at ambient temperature (23°C).

Additionally, the present invention provides a particularly efficient method for incorporating a desired polypeptide into a biodegradable microparticle by first preparing the polypeptide of interest in a highly concentrated, viscous form and carrying out microparticle formation at lowered, yet not freezing, temperatures. The method allows increased amounts of the protein of interest, e.g., 90% of more of the protein provided, to be incorporated into the microparticle.

Accordingly, in one embodiment, the invention is directed to a method of making a biodegradable microparticle comprising:

(a) preparing a polypeptide in a highly concentrated viscous form;

(b) combining the polypeptide with a polymer selected from the group consisting of a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polycaprolactone, a polyorthoester and a polyanhydride, wherein the polymer is present at a concentration of about a 1%-30% in an organic solvent and further wherein the polypeptide is present at .1% to about 40% (w/w);

(c) emulsifying the polymer/IGF-1 solution to form an emulsion;

(d) adding an emulsion stabilizer to the emulsion under conditions that allow microparticles to form;

(e) removing organic solvent from the stabilized emulsion; and

(f) recovering the microparticles.

In another embodiment, the invention is directed to a method of making a biodegradable microparticle comprising:

(a) preparing an IGF-1 or IGF-1 analog composition at a pH of about pH 5.5 to about pH 6.0, in a highly concentrated viscous syrup;

(b) cooling the IGF-1 or IGF-1 analog composition to a temperature of about 2°C to about 8°C;

(c) combining the cooled IGF-1 or IGF-1 analog composition with a poly(α-hydroxy acid) polymer selected from the group consisting of poly(L-lactide), poly(D,L-lactide) and poly(D,L-lactide-co-glycolide), wherein the polymer is present at a concentration of about 5%-20% in dimethylchloride and further wherein the IGF-1 is present at about 3% to about 20% (w/w);

(d) emulsifying the polymer/IGF-1 solution at a temperature of about 2°C to about 8°C;

(e) adding polyvinyl alcohol as an emulsion stabilizer to the polymer/IGF-1 emulsion under conditions that allow microparticles to form;

(f) removing organic solvent from the stabilized polymer/IGF-1 emulsion; and

(g) recovering the microparticles.

In yet other embodiments, the invention is directed to microparticles made using the above methods and sustained-release formulations comprising the microparticles.

In still a further embodiment, the invention is directed to a method of delivering IGF-1 or an IGF-1 analog to a vertebrate subject comprising administering to the vertebrate subject a pharmaceutically effective amount of the sustained-release formulations above.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figure 1 is a graphical release profile of IGF-1 from PLG microparticles containing an IGF-1 load of 20% (squares), 17.8% (triangles) and 19% (solid circles).

Figure 2 is a graphical release profile of IGF-1 from PLG microparticles containing an IGF-1 load of 3.15% (squares), 3.22% (circles) and 3.38% (triangles).

Figure 3 shows the serum concentrations of IGF-1 over time from *in vivo* release studies in animals administered microparticles containing 17.7% w/w IGF-1.

Figure 4 shows the serum concentrations of IGF-1 over time from control animals administered IGF-1 without microparticles.

Figure 5 shows the serum concentrations of IGF-1 over time from *in vivo* release studies in animals administered microparticles containing 3.17% w/w IGF-1.

Figure 6 shows the serum concentrations of IGF-1 for the first six hours from *in vivo* release studies in animals administered microparticles containing 3.17% w/w IGF-1.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A.L. Lehninger, *Biochemistry* (Worth Publishers, Inc., 1975); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd

Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990).

I. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The term "insulin-like growth factor-1" or "IGF-1" as used herein refers to a compound having the primary, secondary and/or tertiary molecular structure of native IGF-1, and which has at least one IGF-1 activity including activity as measured in standard IGF-1 bioassays and/or the ability to bind IGF receptors. The IGF-1 molecule may include posttranslational modifications, such as glycosylation, acetylation, phosphorylation, etc. Furthermore, the term intends salts and other derivatized forms of IGF-1, which serve to render the IGF-1 less soluble, as described further below.

Additionally, for purposes of the present invention, an IGF-1 may be derived from any of several tissues of any mammalian source, such as human, bovine, canine, equine, ovine, porcine, etc. The IGF-1 compound may be purified directly from the source organism, or may be recombinantly or synthetically produced (see further below).

The term "IGF-1 analog" refers to biologically active derivatives or fragments of IGF-1 that retain IGF-1 activity and/or the ability to bind IGF receptors. Such compounds may include amino acid additions, substitutions (generally conservative in nature) and deletions, relative to the native molecule, so long as the modifications do not destroy IGF-1 activity including activity as measured in

standard IGF-1 bioassays and/or the ability of the molecule to bind to IGF receptors. Representative assays include known radioreceptor assays using placental membranes (see, e.g., U.S. Patent No. 5,324,639; Hall et al., *J. Clin. Endocrinol. and Metab.* (1974) 39:973-976; and Marshall et al., *J. Clin. Endocrinol. and Metab.* (1974) 39:283-292), a bioassay that measures the ability of the molecule to enhance incorporation of tritiated thymidine, in a dose-dependent manner, into the DNA of BALB/c 3T3 fibroblasts (see, e.g., Tamura et al., *J. Biol. Chem.* (1989) 262:5616-5621), and the like. Preferably, the analog has at least the same activity as the native molecule.

IGF-1 analogs will generally have at least 60%, preferably 70%, more preferably 80%, preferably 90% to 95% or more, and most preferably 98% or more, amino acid sequence identity to the amino acid sequence of the reference IGF-1 molecule. In general, "identity" refers to an exact amino acid to amino acid correspondence of two or more polypeptide sequences. For example, the IGF-1 analog may have from about 1 to about 20 amino acid substitutions, e.g., 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid substitutions.

Techniques for determining amino acid sequence identity are well known in the art and include comparing the known sequence of IGF-1 to a second amino acid sequence, by e.g., aligning the sequences. Programs available for determining identity between sequences include ALIGN, Dayhoff, M.O. (1978) in *Atlas of Protein Sequence and Structure* 5:Suppl. 3, National biomedical Research Foundation, Washington, DC. and programs in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the GAP program. One of skill in the art can readily use such

programs, along with the default parameters provided by the manufacturer, in order to assess the percent identity between two polypeptides. Other programs for calculating identity or similarity between sequences are known in the art.

The art provides substantial guidance regarding the preparation and use of such analogs, as discussed further below. A fragment of IGF-1 will generally include at least about 10 contiguous amino acid residues of the full-length molecule, preferably about 15-25 contiguous amino acid residues of the full-length molecule, and most preferably about 20-50 or more contiguous amino acid residues of full-length IGF-1. The term "IGF-1 analog" also captures peptides having one or more peptide mimics ("peptoids"), such as those described in International Publication No. WO 91/04282.

Several IGF-1 analogs and fragments are known in the art and include those described in e.g., *Proc. Natl. Acad. Sci. USA* (1986) 83:4904-4907; *Biochem. Biophys. Res. Commun.* (1987) 149:398-404; *J. Biol. Chem.* (1988) 263:6233-6239; *Biochem. Biophys. Res. Commun.* (1989) 165:766-771; Forsberg et al., *Biochem. J.* (1990) 271:357-363; U.S. Patent Nos. 4,876,242 and 5,077,276; International Publication No. WO 87/01038 and WO 89/05822. Representative analogs include one with a deletion of Glu-3 of the mature molecule, analogs with up to five amino acids truncated from the N-terminus, an analog with a truncation of the first three N-terminal amino acids and an analog including the first 17 amino acids of the B chain of human insulin in place of the first 16 amino acids of human IGF-1.

The terms "polypeptide" and "protein" refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus,

peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like.

For purposes of the present invention, the polypeptide entrapped in the microparticle may be one useful in a vaccine or diagnostic and may be derived from any of several known viruses, bacteria, parasites and fungi, as well as any of the various tumor antigens. Alternatively, the polypeptide may be a therapeutic hormone, a transcription or translation mediator, an enzyme, an intermediate in a metabolic pathway, an immunomodulator, and the like.

Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

By "substantially insoluble" is meant that the polypeptide of interest is provided as a composition in which at least about 60% of the polypeptide of interest is not dissolved, more preferably at least about 75%, even more preferably at least about 85% and most preferably, at least about 90% or more of the polypeptide present is not is dissolved. For purposes of the present invention, substantially insoluble polypeptides are typically

provided in the form of a highly concentrated viscous syrup.

By "highly concentrated viscous syrup" or "highly concentrated viscous form" is meant an IGF-1 concentration of at least about 250 mg/ml, for example, at least about 300 mg/ml, or at least about 350 mg/ml, or at least about 425 mg/ml, or about 450 mg/ml to 500 mg/ml, as measured at ambient temperature (23°C). At these concentrations and temperature, this syrup has a density of about 1.0 g/ml to about 1.2 g/ml, more preferably about 1.1 g/ml, and a viscosity of about 13,000 centipoise (cps) to about 19,000 cps, preferably about 14,000 cps to about 18,000 cps, more preferably about 15,000 cps to about 17,000 cps, still more preferably about 15,500 cps to about 16,500 cps, even more preferably about 16,000 cps. In one embodiment, the syrup has an IGF-1 concentration of about 350 mg/ml, a density of about 1.07 g/ml, and a viscosity of about 15,700 cps, as measured at ambient temperature. Density and viscosity are determined using standard techniques well known in the art. See, commonly owned, copending U.S. Patent Application Serial No. 60/096,081, entitled "*Novel IGF-1 Composition and Its Use*," filed August 11, 1998.

The term "microparticle" as used herein, refers to a particle of about 100 nm to about 150 μm in diameter, more preferably about 200 nm to about 30 μm in diameter, and most preferably about 500 nm to about 10 μm in diameter. Preferably, the microparticle will be of a diameter that permits parenteral administration without occluding needles and capillaries. Microparticle size is readily determined by techniques well known in the art, such as photon correlation spectroscopy, laser diffractometry and/or scanning electron microscopy.

Microparticles for use herein will be formed from materials that are sterilizable, non-toxic and biodegradable. Such materials include, without limitation, poly(α-hydroxy acid), polyhydroxybutyric acid, polycaprolactone, polyorthoester, polyanhydride, polyvinyl alcohol and ethylenevinyl acetate. Preferably, microparticles for use with the present invention are derived from a poly(α-hydroxy acid), in particular, from a poly(lactide) ("PLA") or a copolymer of D,L-lactide and glycolide or glycolic acid, such as a poly(D,L-lactide-co-glycolide) ("PLG" or "PLGA"), or a copolymer of D,L-lactide and caprolactone. The microparticles may be derived from any of various polymeric starting materials which have a variety of molecular weights and, in the case of the copolymers such as PLG, a variety of lactide:glycolide ratios, the selection of which will be largely a matter of choice, depending in part on the desired dose of polypeptide and the disorder to be treated. These parameters are discussed more fully below.

As used herein, the term "sustained-release" refers to the release of a polypeptide such as IGF-1 from microparticles over a defined period of time in a continuous, discontinuous, linear or nonlinear manner. For example, release may be essentially biphasic, i.e., the release will include an initial burst of polypeptide from the microparticle, followed by continuous release of the polypeptide from the microparticle over time. Methods of measuring release of a protein from a microparticle over time are well known in the art. See, e.g., Hora et al., *Pharm. Res.* (1990) 7:1190-1194; Hora et al., *Bio/Technology* (1990) 8:755-758; and the examples herein.

The terms "effective amount" or "pharmaceutically effective amount" of polypeptide, as provided herein, refer to a nontoxic but sufficient

amount of the polypeptide to provide the desired biological result. That result can be reduction and/or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. Such amounts are described below. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation.

As used herein, "treatment" refers to both the prevention of the disease in question and the reduction or elimination of symptoms. Treatment may be effected prophylactically (prior to disease symptoms) or therapeutically (following disease symptoms).

By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material which is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the microparticle formulations without causing any undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

By "physiological pH" or a "pH in the physiological range" is meant a pH in the range of approximately 7.2 to 8.0 inclusive, more typically in the range of approximately 7.2 to 7.6 inclusive.

By "vertebrate subject" is meant any member of the subphylum cordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and

other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered.

II. Modes of Carrying Out the Invention

The present invention is based on an efficient and reproducible method of incorporating adequate amounts of a polypeptide of interest into a biodegradable microparticle such that a wide array of disorders may be treated. Particularly, the methods of the present invention provide for the entrapment of polypeptides, provided in a highly concentrated viscous form, within microparticles by forming the microparticles at lowered, but not freezing, temperatures. The method allows for a high percentage of the provided polypeptide, i.e., as high as 90% or more, to become incorporated in the biodegrable microparticle.

The present invention also provides IGF-1 microparticles which allow for the controlled release of polypeptides, such as biologically active IGF-1, for prolonged periods of time. Generally, release is biphasic, with an initial burst of polypeptide from the microparticle, followed by continuous release of the polypeptide over time.

Although the methods of the present invention have been illustrated using IGF-1, almost any protein of therapeutic value may be encapsulated in microparticles using the techniques described herein. For example, the methods of the present invention will find use for encapsulation of a wide variety of substances, including peptides which act as antibiotics and antiviral agents, e.g., immunogenic peptides for use in vaccines and diagnostics; antineoplastics; immunomodulators, such as any of the

various cytokines including interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-12, beta-interferon and gamma-interferon; peptide hormones such as insulin, proinsulin, growth hormone, GHRH, LHRH, EGF, somatostatin, SNX-111, BNP, insulinotropin, ANP, FSH, LH, PSH and hCG, gonadal steroid hormones (androgens, estrogens and progesterone), thyroid-stimulating hormone, inhibin, cholecystokinin, ACTH, CRF, dynorphins, endorphins, endothelin, fibronectin fragments, galanin, gastrin, insulinotropin, glucagon, GTP-binding protein fragments, guanylin, the leukokinins, magainin, mastoparans, dermaseptin, systemin, neuromedins, neurotensin, pancreastatin, pancreatic polypeptide, substance P, secretin, thymosin, and the like; and growth factors, such as PDGF, EGF, KGF, IGF-2, FGF, and the like.

More particularly, proteins for use in vaccines and diagnostics may be of viral, bacterial, fungal or parasitic origin, including but not limited to, those encoded by human and animal viruses and can correspond to either structural or non-structural proteins. For example, the present methods will find use with a wide variety of proteins from the herpesvirus family, including proteins derived from herpes simplex virus (HSV) types 1 and 2, such as HSV-1 and HSV-2 glycoproteins gB, gD and gH; proteins derived from varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) including CMV gB and gH; and proteins derived from other human herpesviruses such as HHV6 and HHV7. (See, e.g. Chee et al., *Cytomegaloviruses* (J.K. McDougall, ed., Springer-Verlag 1990) pp. 125-169, for a review of the protein coding content of cytomegalovirus; McGeoch et al., *J. Gen. Virol.* (1988) 69:1531-1574, for a discussion of the various HSV-1 encoded proteins; U.S.

Patent No. 5,171,568 for a discussion of HSV-1 and HSV-2 gB and gD proteins and the genes encoding therefor; Baer et al., *Nature* (1984) 310:207-211, for the identification of protein coding sequences in an EBV genome; and Davison and Scott, *J. Gen. Virol.* (1986) 67:1759-1816, for a review of VZV.)

Proteins from the hepatitis family of viruses, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), the delta hepatitis virus (HDV), hepatitis E virus (HEV) and hepatitis G virus (HGV), can also be conveniently used in the techniques described herein. By way of example, the viral genomic sequence of HCV is known, as are methods for obtaining the sequence. See, e.g., International Publication Nos. WO 89/04669; WO 90/11089; and WO 90/14436. The HCV genome encodes several viral proteins, including E1 (also known as E) and E2 (also known as E2/NSI). (See, Houghton et al., *Hepatology* (1991) 14:381-388, for a discussion of HCV proteins, including E1 and E2.)

These proteins, as well as antigenic fragments thereof, will find use in the present methods. Similarly, the sequence for the δ-antigen from HDV is known (see, e.g., U.S. Patent No. 5,378,814) and this protein can also be conveniently used in the present methods. Additionally, antigens derived from HBV, such as the core antigen, the surface antigen, sAg, as well as the presurface sequences, preS1 and preS2 (formerly called preS), as well as combinations of the above, such as sAg/preS1, sAg/preS2, sAg/preS1/preS2, and preS1/preS2, will find use herein. See, e.g., "HBV Vaccines - from the laboratory to license: a case study" in Mackett, M. and Williamson, J.D., *Human Vaccines and Vaccination*, pp. 159-176, for a discussion of HBV structure; and U.S. Patent Nos. 4,722,840, 5,098,704, 5,324,513; Beames et al., *J.*

Virol. (1995) 69:6833-6838, Birnbaum et al., *J. Virol.* (1990) 64:3319-3330; and Zhou et al., *J. Virol.* (1991) 65:5457-5464.

Proteins derived from other viruses will also find use in the claimed methods, such as without limitation, proteins from members of the families Picornaviridae (e.g., polioviruses, etc.); Caliciviridae; Togaviridae (e.g., rubella virus, dengue virus, etc.); Flaviviridae; Coronaviridae; Reoviridae; Birnaviridae; Rhabodoviridae (e.g., rabies virus, etc.); Filoviridae; Paramyxoviridae (e.g., mumps virus, measles virus, respiratory syncytial virus, etc.); Orthomyxoviridae (e.g., influenza virus types A, B and C, etc.); Bunyaviridae; Arenaviridae; Retroviradae (e.g., HTLV-I; HTLV-II; HIV-1 (also known as HTLV-III, LAV, ARV, hTLR, etc.)), including but not limited to antigens from the isolates HIV_{IIIb}, HIV_{SF2}, HIV_{LAV}, HIV_{LAI}, HIV_{MN}); HIV-1_{CM235}, HIV-1_{US4}; HIV-2; simian immunodeficiency virus (SIV) among others. See, e.g. Virology, 3rd Edition (W.K. Joklik ed. 1988); *Fundamental Virology*, 2nd Edition (B.N. Fields and D.M. Knipe, eds. 1991), for a description of these and other viruses.

For example, the invention may be used to entrap the gp120 envelope protein from any of the above HIV isolates. The gp120 sequences for a multitude of HIV-1 and HIV-2 isolates, including members of the various genetic subtypes of HIV, are known and reported (see, e.g., Myers et al., Los Alamos Database, Los Alamos National Laboratory, Los Alamos, New Mexico (1992); Myers et al., *Human Retroviruses and Aids, 1990*, Los Alamos, New Mexico: Los Alamos National Laboratory; and Modrow et al., *J. Virol.* (1987) 61:570-578, for a comparison of the envelope sequences of a variety of HIV isolates) and sequences derived from any of these isolates will find

use in the present methods. Furthermore, the invention is equally applicable to other immunogenic proteins derived from any of the various HIV isolates, including any of the various envelope proteins such as gp160 and gp41, gag antigens such as p24gag and p55gag, as well as proteins derived from the pol region.

The present invention will also find use with influenza virus proteins. Specifically, the envelope glycoproteins HA and NA of influenza A are of particular interest for generating an immune response. Numerous HA subtypes of influenza A have been identified (Kawaoka et al., *Virology* (1990) 179:759-767; Webster et al., "Antigenic variation among type A influenza viruses," p. 127-168. In: P. Palese and D.W. Kingsbury (ed.), *Genetics of influenza viruses*. Springer-Verlag, New York). Thus, proteins derived from any of these isolates can also be used in the techniques described herein.

Furthermore, the methods described herein provide a means for entrapping proteins useful for treating a variety of malignant cancers, such as a wide variety of tumor antigens which in turn may be used to mount both humoral and cell-mediated immune responses to particular proteins specific to the cancer in question, such as an activated oncogene, a fetal antigen, or an activation marker. Such tumor antigens include any of the various MAGEs (melanoma associated antigen E), including MAGE 1, 2, 3, 4, etc. (Boon, T. *Scientific American* (March 1993):82-89); any of the various tyrosinases; MART 1 (melanoma antigen recognized by T cells), mutant ras; mutant p53; p97 melanoma antigen; CEA (carcinoembryonic antigen), among others.

It is readily apparent that the subject methods can be used to entrap a variety of proteins

useful for the prevention, treatment and/or diagnosis of a wide variety of diseases.

Polypeptides for use in the subject methods, can be produced in any number of ways which are well known in the art. For example, the polypeptides can be isolated directly from a tissue or organ that produces the same. In the case of IGF-1, the polypeptide can be isolated from blood, such as from serum or plasma, by known methods. See, e.g., U.S. Patent No. 4,769,361; Svoboda et al., *Biochemistry* (1980) 19:790-797; Cornell and Boughdady, *Prep. Biochem.* (1982) 12:57; and Cornell and Boughdady, *Prep. Biochem.* (1984) 14:123.

Alternatively, polypeptides for use in the subject methods can be synthesized chemically, by any of several techniques that are known to those skilled in the peptide art. See, e.g., J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis* (Pierce Chemical Co., Rockford, IL 1984) and G. Barany and R. B. Merrifield, *The Peptides: Analysis, Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, (Academic Press, New York, 1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of Peptide Synthesis*, (Springer-Verlag, Berlin 1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology*, Vol. 1, for classical solution synthesis. The polypeptides of the present invention can also be chemically prepared by the method of simultaneous multiple peptide synthesis. See, e.g., Houghten *Proc. Natl. Acad. Sci. USA* (1985) 82:5131-5135; U.S. Patent No. 4,631,211.

Preferably, the polypeptides are obtained using recombinant methods well known in the art. See, e.g., Sambrook et al., *supra*. For example, the recombinant production of IGF-1 in bacterial and yeast hosts and purification therefrom has been described.

See, e.g., International Publication Nos. WO 96/40776, WO 96/07744, WO 95,06059, WO 95/06064, WO 95/16777, WO 93/11240 and WO 92/04363; EP 567,554 B; U.S. Patent Nos. 5,650,496, 5,612,198, 5,407,810, 5,410,026, 5,288,931, 5,324,639 and 5,231,178; Chang and Swartz, *Protein Folding: in vivo and in vitro* (American Chemical Society, 1993) pp. 178-188; Elliott et al., *J. Protein Chem.* (1990) 9:95-104.

In particular, IGF-1 can be produced in methylotrophic yeast transformants, such as in a protease deficient *P. pastoris* strain as well as in *Saccharomyces cerevisiae* (see, e.g., U.S. Patent Nos. 5,231,178, 5,324,639, 5,612,198 and 5,650,496; International Publication Nos. WO 96/40776, WO 96/07744, WO 92/04363; and EP 567,554 B).

The IGF-1 will either be secreted, if appropriate leader sequences are used, or produced intracellularly and the cells manipulated to allow proper isolation of an IGF-containing product. Particularly preferred methods for producing IGF-1, e.g. in yeast, generally utilize a secretion leader, such as a leader sequence derived from the yeast α-factor signal sequence, as described in EP 128 733. Production in yeast generally includes a fermentation step for cell amplification, followed by purification and refolding to obtain an authentic, properly folded protein. Methods for fermenting the culture, purification and refolding are well known in the art. See, e.g., U.S. Patent Nos. 5,324,639 and 5,650,496, and International Publication Nos. WO 96/07744 and WO 96/40776.

Once obtained, the polypeptide is prepared in a highly concentrated, viscous form for subsequent entrapment within microparticles in accordance with the method of the present invention. Entrapment of the polypeptide in this viscous state allows for

controlled release of the polypeptide from the microparticles over prolonged periods of time. The viscous form of the polypeptide may be prepared using any method that reduces solubility of the polypeptide. Protein solubility may be reduced using any number of techniques well known in the art. Preferably, the method used will not affect biological activity of the polypeptide when released from the microparticles.

In the case of IGF-1, a highly concentrated viscous form of this protein may be prepared by reducing solubility using the methods disclosed in detail in commonly owned, copending U.S. Patent Application Serial No. 60/096,081, entitled "*Novel IGF-1 Composition and Its Use*," filed, August 11, 1998. This highly concentrated form of IGF-1 has a concentration of at least about 250 mg/ml and has the consistency of a viscous syrup. The IGF-1 syrup has a low concentration of salt, and the IGF-1 is biologically active without the need for refolding. Thus, when the entrapped IGF-1 is released from the microparticles into a physiological setting, it retains biological activity similar to that of IGF-1 that has not been rendered in this viscous form.

The first of these methods comprises reducing the solubility of IGF-1 such that IGF-1 is precipitated from a buffer solution containing the IGF-1. Precipitation is achieved by adjusting the pH of the IGF-1-containing buffer solution to a pH above about pH 5.0. Accordingly, IGF-1 is prepared in a buffer solution having an initial pH of less than about pH 5.0, preferably about pH 2.0 to about pH 5.0, more preferably about pH 3.0 to about pH 4.5, even more preferably about pH 3.5 to about pH 4.0. The initial concentration of IGF-1 in this low pH buffer solution will determine the amount of the highly concentrated IGF-1 syrup obtained following upward

adjustment of pH. Thus, a higher initial concentration of IGF-1 will yield a greater amount of precipitated IGF-1 syrup. Regardless of the initial concentration of IGF-1, the concentration of the precipitated IGF-1 is at least about 250 mg/ml as noted above.

In order to obtain this highly concentrated IGF-1 syrup, the initial pH of the buffer solution containing IGF-1 is adjusted upward to a final pH greater than about pH 5.0, preferably to a pH of greater than about 5.0 to about 9.0, more preferably to a pH of greater than about 5.0 to about 8.0, still more preferably to a pH of about 5.5 to about 7.0, even more preferably to a pH of about 5.5 to about 6.5, and most preferably to a pH of about 5.5 to about 6.0. As pH is increased, IGF-1 above the solubility limit at the higher pH conditions precipitates, forming a viscous syrup.

The pH of the buffer solution may be adjusted by standard titrating procedures will known in the art, such as with addition of sodium hydroxide. Alternatively, solution pH may be adjusted by dialyzing the initial buffer solution containing IGF-1 against any suitable buffer solution having the desired final pH above pH 5.0 as disclosed above. Such buffers include, for example, inorganic (e.g., phosphate) and organic (e.g., acetate) buffers. In one embodiment of the invention, the IGF-1 buffer solution having an initial pH less than or equal to pH 5.0 is dialyzed against a sodium citrate buffer at pH 6.0.

This highly concentrated IGF-1 syrup can also be prepared using an appropriate solubilizing agent or so-called solubility enhancer. By "solubility enhancer" is intended a compound that includes a guanidinium group and that is capable of

enhancing the solubility of IGF-1 or a variant of IGF-1. Examples of such solubilizing compounds include the amino acid arginine, as well as amino acid analogs of arginine that retain the ability to enhance solubility of IGF-1 at pH 5.5 or greater. Such analogs include, without limitation, dipeptides and tripeptides that contain arginine. By "enhancing the solubility" of IGF-1 is meant increasing the amount of IGF-1 that can be dissolved in solution at pH 5.5 or greater, 6.0 or greater, 7.0 or greater, 8.0 or greater, or 9.0 or greater in the presence of a guanidinium-containing compound compared to the amount of IGF-1 that can be dissolved at pH 5.5 or greater, 6.0 or greater, 7.0 or greater, 8.0 or greater, or 9.0 or greater, respectively, in a solution with the same components but lacking in the guanidinium-containing compound. The ability of a guanidinium-containing compound to enhance the solubility of a IGF-1 can be determined using methods well known in the art. In general, the concentration of the solubilizing compound present in the composition is from about 10 mM to about 1 M, and, for example, in the case of the compound arginine, in a concentration range of about 20 mM to about 200 mM, as disclosed in the commonly owned, copending U.S. Application Serial No. 60/064,891, filed November 7, 1997.

In this manner, addition of a solubility enhancer to the solution allows for the preparation of a highly concentrated IGF-1 solution. The solubility enhancer is then removed from this IGF-1 solution by dialysis or diafiltration. Removal of the solubility enhancer results in precipitation of IGF-1 in the highly concentrated syrup form. The soluble portion of IGF-1 can then be decanted off, and the IGF-1 syrup recovered.

An alternative method for reducing solubility of polypeptide such as IGF-1 involves complexing the protein with a divalent metal, such as Cu++, Mn++, Ni++, Zn++ and/or Fe++. Methods for complexing proteins with such metals are known in the art. See, e.g., Johnson et al., *Nature Med.* (1996) 2:795.

Additionally, solubility of polypeptides may be reduced by forming acid addition salts (formed with the free amino groups of the polypeptide) with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like. Furthermore, the polypeptides can be provided in the form of a pamoate, tannate, stearate or palmitate salt. See, e.g., U.S. Patent No. 5,134,122.

Protein solubility can also be reduced using biological cations (see, e.g., International Publication No. WO 92/11844) or complexing agents such as albumin or protamine, at ratios of about 1:10 to about 4:1, complexing agent to IGF-1.

It is important to note that the present invention distinguishes from prior techniques because the polypeptide itself is rendered insoluble, rather than by addition of gelling agents to the polypeptide/polymer solution.

Additionally, the polypeptides can be formulated with protein stabilizers in order to preserve the activity thereof. Such stabilizers are known in the art and include, e.g., simple salts,

buffer salts, polyhdroxylated compounds such as glycerol, mannitol, sucrose and polyethylene glycols, and surfactants. See, e.g., International Publication No. WO 92/11844.

After the polypeptide is prepared in a highly concentrated, viscous form, it is combined with appropriate polymers to form microparticles for subsequent delivery, as described further below. Prior to doing so, the polypeptide may be cooled to a temperature of approximately 1°C to about 20°C, more preferably about 2°C to about 10°C, even more preferably about 2°C to about 8°C, and most preferably to about 4°C.

Biodegradable polymers for manufacturing microparticles useful in the present invention are readily commercially available from, e.g., Boehringer Ingelheim, Germany and Birmingham Polymers, Inc., Birmingham, AL. For example, useful polymers for forming the microparticles herein include those derived from: polyhydroxybutyric acid; polycaprolactone; polyorthoester; polyanhydride; polyvinyl alcohol; ethylenevinyl acetate; as well as a poly(α-hydroxy acid), such as poly(L-lactide), poly(D,L-lactide) (both known as "PLA" herein), poly(hydoxybutyrate), copolymers of D,L-lactide and glycolide, such as poly(D,L-lactide-co-glycolide) (designated as "PLG" or "PLGA" herein) or a copolymer of D,L-lactide and caprolactone.

Particularly preferred polymers for use herein are PLA and PLG polymers. These polymers are available in a variety of molecular weights, and the appropriate molecular weight to provide the desired release rate for the polypeptide in question is readily determined by one of skill in the art. Thus, e.g., for PLA, a suitable molecular weight will be on the order of about 2000 to 250,000. For PLG, suitable

molecular weights will generally range from about 10,000 to about 200,000, preferably about 15,000 to about 150,000, and most preferably about 50,000 to about 100,000.

If a copolymer such as PLG is used to form the microparticles, a variety of lactide:glycolide ratios will find use herein and the ratio is largely a matter of choice, depending in part on the rate of degradation desired. For example, a 50:50 PLG polymer, containing 50% D,L-lactide and 50% glycolide, will provide a fast resorbing copolymer while 75:25 PLG degrades more slowly, and 85:15 and 90:10, even more slowly, due to the increased lactide component. It is readily apparent that a suitable ratio of lactide:glycolide is easily determined by one of skill in the art based on the nature disorder to be treated. Moreover, mixtures of microparticles with varying lactide:glycolide ratios will find use in the formulations in order to achieve the desired release kinetics. PLG copolymers with varying lactide:glycolide ratios and molecular weights are readily available commercially from a number of sources including from Boehringer Ingelheim, Germany and Birmingham Polymers, Inc., Birmingham, AL. These polymers can also be synthesized by simple polycondensation of the lactic acid component using techniques well known in the art, such as described in Tabata et al., *J. Biomed. Mater. Res.* (1988) 22:837-858.

The microparticles are prepared using any of several methods well known in the art, the critical parameter being that the highly concentrated, viscous form of the protein is added to the polymer solution at a low temperature, as explained above. For example, double emulsion/solvent evaporation techniques, such as described in U.S. Patent No.

3,523,907 and Ogawa et al., *Chem. Pharm. Bull.* (1988) 36:1095-1103, can be used herein to form the microparticles. These techniques involve the formation of a primary emulsion consisting of droplets of polymer solution containing the IGF-1, which is subsequently mixed with a continuous aqueous phase containing a particle stabilizer/surfactant.

More particularly, a water-in-oil-in-water (w/o/w) solvent evaporation system can be used to form the microparticles, as described by O'Hagan et al., *Vaccine* (1993) 11:965-969 and Jeffery et al., Pharm. Res. (1993) 10:362. In this technique, the particular polymer is combined with an organic solvent, such as ethyl acetate, dimethylchloride (also called methylene chloride and dichloromethane), acetonitrile, acetone, chloroform, and the like. The polymer will be provided in about a 1%-30% (w/v) solution, more preferably about a 3%-25% solution and most preferably, about a 5%-20% solution, in organic solvent. An amount of the desired polypeptide preparation will be added to the polymer solution to provide a ratio of polymer:polypeptide from about 60:40 to about 99.9:0.1, preferably about 75:25, and most preferably about 97:3. The polymer/polypeptide solution is emulsified using e.g, an homogenizer. The emulsion is then combined with a larger volume of an aqueous solution of an emulsion stabilizer such as polyvinyl alcohol (PVA) or polyvinyl pyrrolidone. The emulsion stabilizer is typically provided in about a 2-15% solution, more typically about a 4-10% solution. The mixture is then homogenized to produce a stable w/o/w double emulsion. Organic solvents are then evaporated.

The formulation parameters can be manipulated to allow the preparation of small (<5μm) and large (>30μm) microparticles. See, e.g., Jeffery

et al., *Pharm. Res.* (1993) 10:362-368; McGee et al., *J. Microencap.* (1996). For example, reduced agitation results in larger microparticles, as does an increase in internal phase volume. Small particles are produced by low aqueous phase volumes with high concentrations of PVA.

Microparticles can also be formed using spray-drying and coacervation as described in, e.g., Thomasin et al., *J. Controlled Release* (1996) 41:131; U.S. Patent No. 2,800,457; Masters, K. (1976) *Spray Drying* 2nd Ed. Wiley, New York; air-suspension coating techniques, such as pan coating and Wurster coating, as described by Hall et al., (1980) The "Wurster Process" in *Controlled Release Technologies: Methods, Theory, and Applications* (A.F. Kydonieus, ed.), Vol. 2, pp. 133-154 CRC Press, Boca Raton, Florida and Deasy, P.B., *Crit. Rev. Ther. Drug Carrier Syst.* (1988) S(2):99-139; and ionic gelation as described by, e.g., Lim et al., *Science* (1980) 210:908-910.

In the above methods, emulsification is carried out at lowered temperatures, but not freezing, in order to maintain a viscous, gel-like emulsion. Generally, the emulsification procedure will be carried out at a temperature of approximately 1°C to about 20°C, more preferably about 2°C to about 10°C, even more preferably about 2°C to about 8°C, and most preferably to about 4°C. The proper conditions for achieving the desired viscosity can be readily determined by one of skill in the art.

Degradation of the particles is by hydrolysis of ester linkages in the backbone. Therefore, the rate of degradation may be controlled by changing polymer properties that influence water uptake, such as by adjusting the hydrophilicy and crystallinity of the particle which, in turn, determines the rate of water penetration. As

explained above, one way of controlling the degradation rate is by adjusting the monomer ratio (e.g., lactide:glycolide). Degradation rate may also be controlled by such factors as the particular monomer used (e.g., L-lactide versus D,L-lactide), polymer molecular weight, as well as the presence of polymer degradation modifiers and pore forming agents, all well known in the art. See, e.g., International Publication No. WO 94/12158. Degradation rate may also be controlled by the amount of polypeptide present in the microparticles (see further below for appropriate amounts).

Thus, for example, a microparticle formed of poly(L-lactide), or a high molecular weight poly(lactide-co-glycolide) polymer with low glycolide amounts, will exhibit slow erosion and cause release of the polypeptide to be governed largely by diffusion. On the other hand, water uptake and hydrolysis of the polymer can be enhanced by increasing the glycolide concentration and lowering the molecular weight. Additionally, hydrophilic excipients such as salts, carbohydrates and surfactants can also be incorporated to increase water penetration into the microparticles thereby accelerating erosion of the polymer. Pore forming agents include substances that add microstructure to the particles, for example, water soluble compounds such as inorganic salts and sugars, present in the range of about 1% to about 30% (w/w polymer). One of skill in the art can readily vary the above parameters in order to produce a microparticle with desired degradation characteristics.

In general, a microparticle which delivers a polypeptide over a period of at least about 24 hours up to 2-3 months or more, more preferably over a period of at least about 1 week, and even more

preferably over a period of about 2-4 weeks or more, is desirable. Methods of measuring release of a protein from a microparticle over time are well known in the art. See, e.g., Hora et al., *Pharm. Res.* (1990) 7:1190-1194; Hora et al., *Bio/Technology* (1990) 8:755-758; and the examples herein.

Particle size can be determined by, e.g., laser light scattering, using for example, a spectrometer incorporating a helium-neon laser. Generally, particle size is determined at room temperature and involves multiple analyses of the sample in question (e.g., 5-10 times) to yield an average value for the particle diameter. Particle size is also readily determined using scanning electron microscopy (SEM).

Prior to use of the microparticles, protein content is generally determined so that an appropriate amount of the microparticles may be delivered to the subject in order to elicit an appropriate biological response. Protein content of the microparticles can be determined according to methods known in the art, such as by disrupting the microparticles and extracting the entrapped polypeptide. For example, microparticles can be dissolved in dimethylchloride and the protein extracted into distilled water, as described in, e.g., Cohen et al., *Pharm. Res.* (1991) 8:713; Eldridge et al., *Infect. Immun.* (1991) 59:2978; and Eldridge et al., *J. Controlled Release* (1990)11:205. Alternatively, microparticles can be dispersed in 0.1 M NaOH containing 5% (w/v) SDS. The sample is agitated, centrifuged and the supernatant assayed for the particular polypeptide using an appropriate assay. See, e.g., O'Hagan et al., *Int. J. Pharm.* (1994) 103:37-45.

For purposes of the present invention, preferably the particles comprise from about .1% to

about 40% (w/w) polypeptide, more preferably about 2% to about 25% (w/w) polypeptide, and even more preferably about 3%-4% to about 18%-20% (w/w) polypeptide. The load of polypeptide in the microparticles will depend on the desired dose and the condition being treated, as discussed in more detail below.

Once formulated, the microparticles of the present invention are generally combined with a pharmaceutically acceptable excipient or vehicle, including liquids such as water, saline, glycerol, polyethyleneglycol, hyaluronic acid, ethanol, etc. Suitable excipients for nonliquid formulations are also known to those of skill in the art. Pharmaceutically acceptable salts can be used in the compositions of the present invention and include, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients and salts is available in *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990).

Additionally, auxiliary substances, such as wetting or emulsifying agents, biological buffering substances, surfactants, and the like, may be present in such vehicles. A biological buffer can be virtually any solution which is pharmacologically acceptable and which provides the formulation with the desired pH, i.e., a pH in the physiologically acceptable range. Examples of buffer solutions include saline, phosphate buffered saline, Tris buffered saline, Hank's buffered saline, and the like.

Once formulated, the compositions of the invention are generally administered parenterally.

Administration can include, for example, administration intravenously, intra-arterially, intra-articularly (e.g., into the knee), subcutaneously, intradermally, intramuscularly, transdermally, intranasally, mucosally, and by aerosol administration. For example, the composition can be administered by inhalation, e.g., as a nasal or mouth spray or aerosol. The compositions may also be delivered *in situ*, e.g., by implantation.

A pharmaceutically or therapeutically effective amount of the polypeptide of interest will be delivered to the subject. The precise effective amount will vary from subject to subject and will depend upon the species, age, the subject's size and health, the nature and extent of the condition being treated, recommendations of the treating physician, and the therapeutics or combination of therapeutics selected for administration. Thus, the effective amount for a given situation can be determined by routine experimentation. For purposes of the present invention, generally a therapeutic amount will be in the range of about 0.1 μg/kg to about 100 mg/kg, more preferably about 1 μg/kg to about 1 mg/kg, and most preferably about 2 μg/kg to about 100 μg/kg, in at least one dose. The subject may be administered as many doses as is required to reduce and/or alleviate the signs, symptoms, or causes of the disorder in question, or bring about any other desired alteration of a biological system.

The compositions can be used for a variety of purposes, depending on the polypeptide entrapped in the microparticle. For example, for IGF-1, the compositions of the present invention may be used to e.g., stimulate growth of cells *in vitro* or *in vivo* in a number of tissues and cell types. The compositions can also be used for bone repair and replacement

therapy, to treat osteoporosis or osteoarthritis, to inhibit an inflammatory response, ischemic injury, and organ rejection upon transplantation, to treat pancreatic, liver, kidney, nerve and cardiac disorders, and to increase lactation and meat production in cattle and other farm animals.

Not only can the microparticles be used therapeutically, as described above, the compositions may also be used as vaccines, in order to generate an immune response, or to prepare antibodies, both polyclonal and monoclonal, for, e.g., diagnostic purposes, as well as for immunopurification of the polypeptide of interest. If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) is immunized with the compositions of the present invention. In order to enhance immunogenicity, an immunological adjuvant is generally used with the compositions and the antigen can be linked to a carrier. Immunization for the production of antibodies is generally performed by injecting the composition parenterally (generally subcutaneously or intramuscularly). The animal is usually boosted 2-6 weeks later with one or more injections of the antigen. Polyclonal antisera is then obtained from the immunized animal and treated according to known procedures. See, e.g., Jurgens et al. (1985) *J. Chrom.* 348:363-370.

Monoclonal antibodies are generally prepared using the method of Kohler and Milstein, *Nature* (1975) 256:495-96, or a modification thereof. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) is removed and dissociated into single cells. If desired, the spleen cells may be screened (after removal of nonspecifically adherent cells) by applying

a cell suspension to a plate or well coated with the protein antigen. B cells, expressing membrane-bound immunoglobulin specific for the antigen, will bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium (e.g., hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the immunizing antigen (and which do not bind to unrelated antigens). The selected monoclonal antibody-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice). See, e.g., M. Schreier et al., *Hybridoma Techniques* (1980); Hammerling *et al.*, *Monoclonal Antibodies and T-cell Hybridomas* (1981); Kennett et al., *Monoclonal Antibodies* (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the polypeptide of interest can be screened for various properties; i.e., for isotype, epitope, affinity, etc.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Example 1

Preparation of rhIGF-1-PLG Microparticles (20% w/w)

Recombinant human IGF-1 (rhIGF-1) for use in these experiments was recombinantly produced in the yeast strain *Pichia pastoris* and purified essentially as described in U.S. Patent Nos. 5,324,639, 5,324,660, 5,650,496 and International Publication No. WO 96/40776. Following isolation, IGF-1 was formulated into microparticles. Materials used to formulate the microparticles were as follows:

(1) 400 mg of the polymer poly(D,L-lactide-co-glycolide) composed of a 50:50 mol ratio of lactide to glycolide with a molecular weight average of 22 Kdal, (Boehringer Ingelheim Resomer RG 502H), was dissolved in 2.5 ml of methylene chloride (DCM, HPLC grade, obtained from Aldrich Chemicals, U.S.A.) to render a 16% PLG solution;

(2) 10% polyvinyl alcohol (PVA) 23,000 MW (Aldrich Chemicals, U.S.A.) in water; and

(3) rhIGF-1, in 0.1 M acetic acid, pH 4.5 (112 mg/ml).

Three separate batches of rhIGF-1 PLG microparticles at a theoretical loading of 20% w/w, were prepared using the following process. Prior to entrapment in the PLG microparticle, the protein was first prepared in a highly concentrated, viscous form by adjusting the pH of a rhIGF-1 buffered solution. This method is described in detail in commonly owned, copending U.S. Patent Application Serial No. 60/096,081, entitled "*Novel IGF-1 Composition and Its Use*," filed, August 11, 1998.

Briefly, preparation by pH adjustment of a rhIGF-1 buffered solution was achieved as follows. 0.5 ml of an rhIGF-1 solution (112 mg/ml) at pH 4.5 was taken and the solubility of the rhIGF-1 was changed by increasing the pH of the solution with 0.1

M sodium hydroxide to 5.5-6.0. This caused the majority of the rhIGF-1 to precipitate as a highly concentrated, opalescent viscous syrup having a concentration of rhIGF-1 of about 350 mg/ml.

Alternatively, the syrup form of rhIGF-1 was prepared by taking bulk rhIGF-1 at a concentration of 13 mg/ml, concentrating it to 74 mg/ml in an initial buffer solution at pH 4.0 and then dialyzing this solution against a 10 mM sodium citrate/140 mM sodium chloride buffer at pH 6.0 using spectra pore tubing 1000 MWCO. The buffer solution was decanted off and the precipitated polypeptide recovered in the form of the viscous syrup.

The rhIGF-1 syrup may also be prepared by removal of a solubility enhancer from a rhIGF-1 solution containing the solubility enhancer as disclosed in commonly owned, copending U.S. Patent Application Serial No. 60/096,081, entitled "*Novel IGF-1 Composition and Its Use,*" filed August 11, 1998. In this manner, arginine is used as a solubility enhancer to prepare a high concentration IGF-1 solution from which the solubilizing agent is removed to precipitate the IGF-1 syrup form of the present invention. For example, rhIGF-1 at 100 mg/ml in 10 mM sodium citrate, 120 mM arginine, pH 6.0 is dialyzed against 10 mM sodium citrate, 140 mM sodium chloride, pH 6.0 at 4°C. Under these conditions, rhIGF-1 is only soluble to about 10 mg/ml. Of the original 100 mg/ml, 90 mg/ml precipitates to form the highly concentrated syrup and 10 mg/ml remains in solution. The soluble portion of the rhIGF-1 syrup can be decanted off and the rhIGF-1 syrup recovered.

The density of the IGF-I syrup was determined by weight at ambient temperature (23°C). 10 ml of IGF-I syrup was prepared volumetrically and its weight determined on a Mettler AE240. the weight

of the 10 ml sample of IGF-I syrup was determined to be 10.7 grams. Therefore, the density of the IGF-I syrup was determined to be 1.07 g/ml.

The viscosity of the IGF-I syrup was determined with a Cannon Instruments LV2000 Rotary Viscometer. The instrument was calibrated with a viscosity standard provided by the manufacturer. All measurements were performed at ambient temperature. The viscosity of the IGF-I syrup was determined to be approximately 15,700 centipoise.

The recovered rhIGF-1 syrup was then encapsulated in PLG microparticles as follows. The rhIGF-1 syrup was cooled to 4°C and 1.4 ml of the PLG solution was added. The mixture was homogenized at 10,000 rpm on an ultra turrex homogenizer for 2.5 minutes at 4°C. This resulted in the formation of a viscous w/o emulsion. This emulsion was poured into 20 ml of the 10% PVA and stirred at 500 rpm on a magnetic stirrer overnight to allow the methylene chloride to evaporate.

The multiple emulsion was then centrifuged at 30,000 G and the pellet which was composed of rhIGF-1-PLG microparticles, recovered and washed twice with distilled water to eliminate free PVA on the surface of the microparticles. The microparticles were freeze-dried and stored in a desiccator.

Example 2

In Vitro Release Profile of rhIGF-1-PLG Microparticles (20% w/w)

The three batches of rhIGF-1 prepared as described in Example 1, were used in an *in vitro* release study at 37°C. Several 3 ml vials, each containing 10 mg of microparticles, were weighed, 1 ml PBS was added to each vial and the vials were kept at 37°C. At each time point indicated, 1 vial was

withdrawn and the supernatant assayed for protein concentration using blank microparticles as control. IGF-1 concentration was estimated using a standard BCA assay (Sigma Chemicals, St. Louis, MO). The cumulative release was plotted versus time (see Figure 1). As is readily seen, sustained, steady release of IGF-1 was achieved for at least three weeks.

Example 3

Analysis of *In Vitro*-Released rhIGF-1

Four time points from the above *in vitro* study, day 1, 7, 14 and 21, were selected to analyze the quality of the rhIGF-1 released from the PLG microparticles. The analysis was carried by three techniques, CN-RP-HPLC, SDS PAGE and a mitogenic bioassay. The mitogenic bioassay was conducted by determining the effect of IGF-1 on proliferating human osteosarcoma MG-63 cells, followed by MTT staining. See, e.g., Lopaczynski et al., *Regulatory Peptides* (1993) 48:207-216. Absorbance was read at 570 nm. Optical density (O.D.) was corrected using activity of a standard IGF-1 in International Units (I.U.). The results are summarized in Tables 1-3.

Table 1
In Vitro Release of rhIGF-1 from
Three PLG/rhIGF-1 Batches CN-RP-HPLC Analysis*

Sample	% Purity	%Main Peak
Std/MICPL002	98.52	92.56
Batch I/Day 1	96.18	89.45
Batch I/Day 7	96.10	89.31
Batch I/Day 14	95.62	88.83
Batch I/Day 21	95.78	89.35
Batch II/Day 1	95.70	88.95
Batch II/Day 7	95.67	88.92
Batch II/Day 14	95.73	88.97
Batch II/Day 21	95.74	89.29
Batch III/Day 1	95.74	88.96
Batch III/Day 7	95.75	88.93
Batch III/Day 14	95.62	88.81
Batch III/Day 21	95.84	89.37

*Peaks area are expressed as a % of the total peak for each sample.

Table 2
In Vitro Release of rhIGF-1 from Three PLG/rhIGF-1 Batches Non-reducing SDS-PAGE Analysis (Colloidal Coomassie)*

Sample	% Monomer	% Dimer	% Others
Std/MICPL002	100	0	0
Batch I/Day 1	100	0	0
Batch I/Day 7	100	0	0
Batch I/Day 14	100	0	0
Batch I/Day 21	100	0	0
Batch II/Day 1	100	0	0
Batch II/Day 7	100	0	0
Batch II/Day 14	100	0	0
Batch II/Day 21	100	0	0
Batch III/Day 1	100	0	0
Batch III/Day 7	100	0	0
Batch III/Day 14	100	0	0
Batch III/Day 21	100	0	0

*Each gel was scanned and gel bands converted to peak intensities. Bands are expressed as a % of the total peak area for each sample. "Other" indicates any peak larger than monomer (dimer, trimer, etc.).

Table 3
In Vitro Release of rhIGF-1 from Three PLG/rhIGF-1 Batches Mitogenic Bioassay*

Sample	IU/mg²	% Activity
Std/MICPLOO2	1220	93.8
Batch I/Day 1	1260	96.9
Batch I/Day 7	1400	107.6
Batch I/Day 14	1280	98.4
Batch I/Day 21	1380	106.1
Batch II/Day 1	1320	101.53
Batch II/Day 7	1090	83.8
Batch II/Day 14	1460	112.3
Batch II/Day 21	1360	104.6
Batch III/Day 1	1320	101.5
Batch III/Day 7	1410	108.4
Batch III/Day 14	1260	96.9
Batch III/Day 21	1880	144.6

*1 mg rhIGF-1 = 1300 IU

As can be seen in Tables 1-3, most of the protein retained the native structure and the IGF-1 remained stable and active over the three weeks. It is especially notable that the protein remained stable in the absence of stabilizing excipients.

Example 4

Preparation of rhIGF-1-PLG Microparticles (4% w/w)

Three separate batches of rhIGF-1 PLG microparticles at a theoretical loading of 4% w/w were prepared using the following process. 0.5 ml of the rhIGF-1 solution described in Example 1 was treated,

also as described in Example 1, to render the rhIGF-1 in the form of a highly concentrated, viscous syrup. The rhIGF-1 syrup was cooled to 4°C and 8.75 ml of the 16% PLG solution in methylene chloride, described in Example 1, was added. The mixture was homogenized as described above. The emulsion was added to 40 ml of 10% PVA and stirred at 500 rpm on a magnetic stirrer overnight to allow the methylene chloride to evaporate.

The multiple emulsion was then centrifuged at 30,000 G and the pellet which was composed of rhIGF-1-PLG microparticles, was recovered and washed twice with distilled water to eliminate free PVA on the surface of the microparticles. The microparticles were freeze-dried and stored in a desiccator.

Example 5

In Vitro Release Profile of rhIGF-1-PLG Microparticles (4% w/w)

The three batches of rhIGF-1 prepared as described in Example 1, were used in an *in vitro* release study at 37°C, as described in Example 2. Release was plotted versus time (see Figure 2). As is readily seen, sustained, steady release of IGF-1 was achieved for at least four weeks.

Example 6

In Vivo Release Studies of rhIGF-1-PLG Microparticles (17.7% w/w)

40 mg/kg of the microparticles containing 17.7% w/w IGF-1, prepared as described in Example 1, were administered subcutaneously to male CD rats. 9 rats were evaluated, 3 rats per sampling point at the times indicated in Figures 3 and 4. 0.5 ml blood was collected from the jugular vein prior to dosing and at each sampling point. Control rats were administered

10 mg/kg IGF-1 in buffer without microparticles. Blood samples were assayed for serum concentrations of IGF-1 using a standard IGF-1 ELISA.

As can be seen in Figure 3, the microparticles provided continuous release of IGF-1 for at least 336 hours (2 weeks). As shown in Figure 4, serum concentrations of IGF-1 in rats given the control formulations dropped to initial levels by 8 hours post-administration. Therefore, microparticles prepared using the techniques of the present invention provide for the controlled release of IGF-1 for prolonged periods of time.

Example 7

In Vivo Release Studies of rhIGF-1-PLG Microparticles (3.17% w/w)

25 mg/kg of the microparticles containing 3.17% w/w IGF-1, prepared as described in Example 4, were administered subcutaneously to male CD rats. 9 rats were evaluated, 3 rats per sampling point at the times indicated in Table 4. Blood samples were collected for 34 days for the analysis of IGF-1 and glucose. Serum IGF-1 concentration was measured by ELISA assay.

A significant burst was observed during the first 6 hours after administration with a mean (±SD) Cmax of 2868±686 ng/ml at 1.5 hours from the 9 rats (Table 4 and Figures 5 and 6). IGF-1 concentrations declined to 77±24 ng/ml at 48 hours and slowly increased to 504±214 ng/ml at 10 days after dosing. No IGF-1 concentrations were detectable (<25 ng/ml) after 18 days. These results indicated that the IGF-1 low-load PLG formulation was associated with an initial burst followed by a sustained release phase which lasted for approximately 18 days in rats. The results were similar to those of the IGF-1 high-load

PLG formulation. However, the burst was greater in this study. Also, the low-load formulation produced higher IGF-1 concentrations in the rats despite a smaller dose (25 mg/kg compared to 40 mg/kg in the high-load formulation study).

The initial burst of IGF-1 concentrations caused significant hypoglycemia in all rats which required IP glucose treatment. However, all rats recovered by six hours. Nodules at the injection sites were observed in a few rats during the first week. These nodules disappeared in week 2.

Table 4
Mean IGF-1 Concentration versus Time Data (n=9)

Time (Hr)	Mean	sd	% cv
0	0	0	0
0.167	1843	944	51
0.417	2377	1044	44
0.667	2573	840	33
1	2732	887	32
1.5	2868	686	24
2	2307	871	38
3	2035	728	36
4	1549	585	38
6	1198	508	42
24	164	53	32
48	77	24	31
96	105	40	38
144	209	60	29
192	394	215	55
240	504	214	42
288	346	89	26
336	202	50	25
384	136	81	60
432	89	74	83

Thus, novel microparticles and methods of making and using the same are disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

We Claim:

1. A method of making a biodegradable microparticle comprising:

(a) preparing a polypeptide in a highly concentrated viscous form;

(b) combining said viscous form of said polypeptide with a polymer selected from the group consisting of a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polycaprolactone, a polyorthoester and a polyanhydride, wherein said polymer is present at a concentration of about 1%-30% in an organic solvent and further wherein said polypeptide is present at .1% to about 40% (w/w);

(c) emulsifying the polymer/polypeptide solution to form an emulsion;

(d) adding an emulsion stabilizer to the emulsion under conditions that allow microparticles to form;

(e) removing organic solvent from the stabilized emulsion; and

(f) recovering the microparticles.

2. The method of claim 1, wherein said polypeptide is an insulin-like growth factor-1 (IGF-1) or an IGF-1 analog.

3. The method of claim 2, wherein said viscous form of said IGF-1 or said IGF-1 analog is cooled to a temperature of about 2°C to about 8°C prior to step (b).

4. The method of claim 3, wherein said viscous form of said IGF-1 or said IGF-1 analog is cooled to a temperature of about 4°C.

5. The method of claim 2, wherein the polymer is a poly(α-hydroxy acid) selected from the group consisting of poly(L-lactide), poly(D,L-lactide) and poly(D,L-lactide-co-glycolide).

6. The method of claim 5, wherein the polymer is poly(D,L-lactide-co-glycolide).

7. The method of claim 2, wherein the IGF-1 or IGF-1 analog present in step (b) is present at about 3% to about 20% (w/w).

8. The method of claim 2, wherein the IGF-1 or IGF-1 analog present in step (b) is present at about 3% to about 4% (w/w).

9. The method of claim 2, wherein the IGF-1 or IGF-1 analog present in step (b) is present at about 18% to about 20% (w/w).

10. The method of claim 1, wherein the organic solvent is dimethylchloride.

11. The method of claim 1, wherein the emulsion stabilizer is polyvinyl alcohol.

12. A method of making a biodegradable microparticle comprising:

(a) preparing an insulin-like growth factor-1 (IGF-1) or an IGF-1 analog in a highly concentrated viscous form;

(b) cooling said viscous form of said IGF-1 or said IGF-1 analog to a temperature of about 2°C to about 8°C;

(c) combining said cooled viscous form of said IGF-1 or said IGF-1 analog with a poly(α-hydroxy

acid) polymer selected from the group consisting of poly(L-lactide), poly(D,L-lactide) and poly(D,L-lactide-co-glycolide), wherein said polymer is present at a concentration of about 5%-20% in dimethylchloride and further wherein said IGF-1 is present at about 3% to about 20% (w/w);

(d) emulsifying the polymer/IGF-1 solution to form an emulsion;

(e) adding polyvinyl alcohol as an emulsion stabilizer to the emulsion under conditions that allow microparticles to form;

(f) removing organic solvent from the stabilized emulsion; and

(g) recovering the microparticles.

13. The method of claim 12, wherein said viscous form of said IGF-1 or said IGF-1 analog is cooled to a temperature of about 4°C.

14. The method of claim 12, wherein the polymer is poly(D,L-lactide-co-glycolide).

15. The method of claim 12, wherein said IGF-1 or said IGF-1 analog present in step (c) is present at about 3% to about 4% (w/w).

16. The method of claim 12, wherein said IGF-1 or said IGF-1 analog present in step (c) is present at about 18% to about 20% (w/w).

17. A biodegradable microparticle produced by the method of any of claims 1-16.

18. A sustained-release formulation comprising the microparticle of claim 17 and a pharmaceutically acceptable excipient.

19. Use of the microparticle according to claim 17 for the manufacture of a sustained-release formulation useful for delivering IGF-1 or an IGF-1 analog to a vertebrate subject.

20. The use of claim 19, wherein the sustained-release formulation is useful for parenteral delivery.

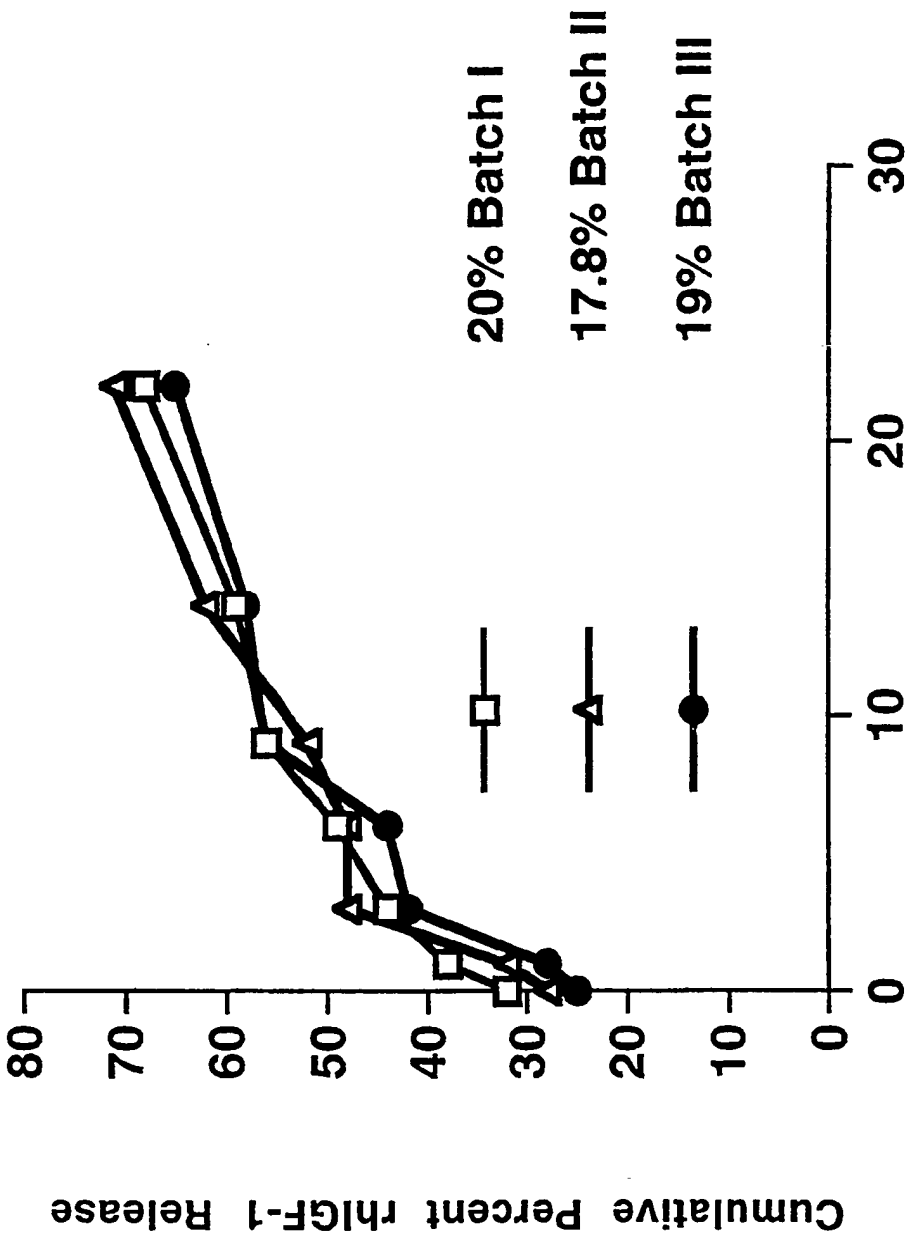

Cumulative Percent rhIGF-1 Release
0
10
20
30
40
50
60
70
80
0
10
20
30
Days
20% Batch I
17.8% Batch II
19% Batch III

FIG. 1

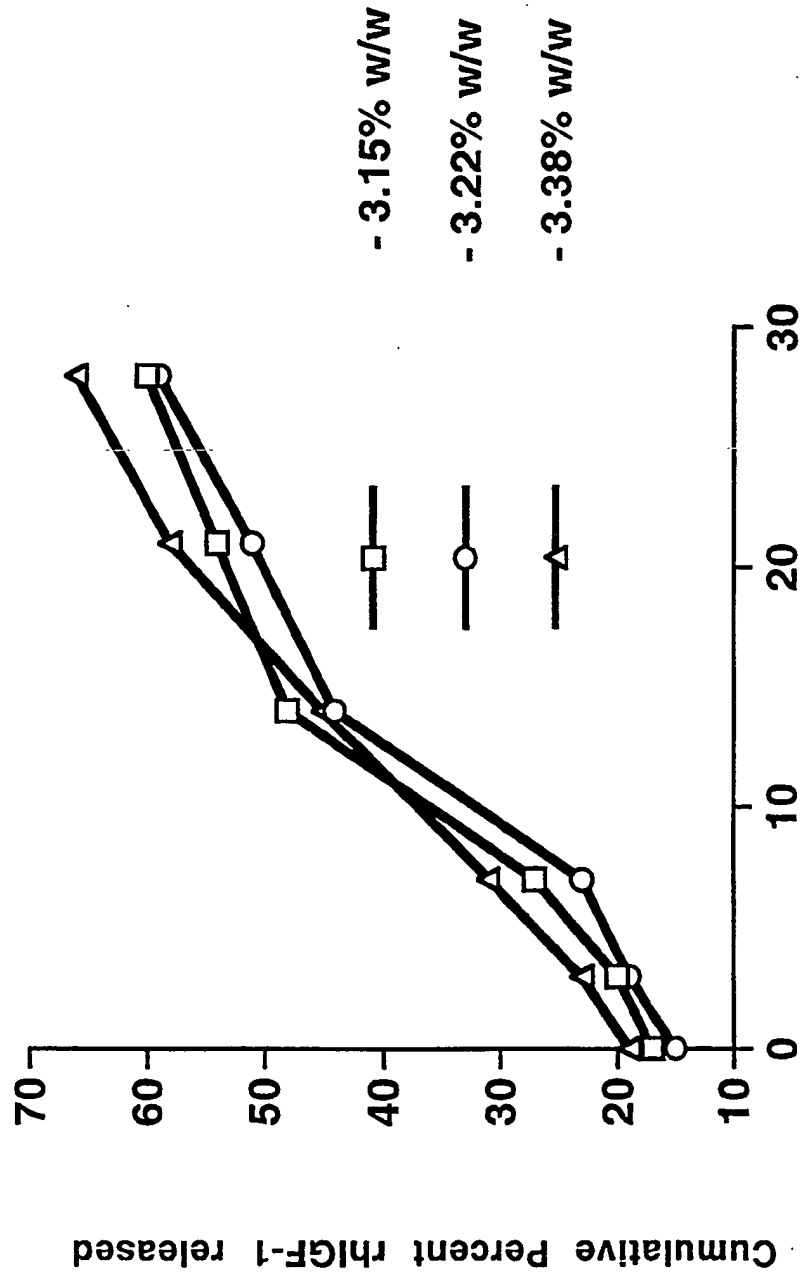

- 3.15% w/w
- 3.22% w/w
- 3.38% w/w
Cumulative Percent rhIGF-1 released
70
60
50
40
30
20
10
0
10
20
30
Days

FIG. 2

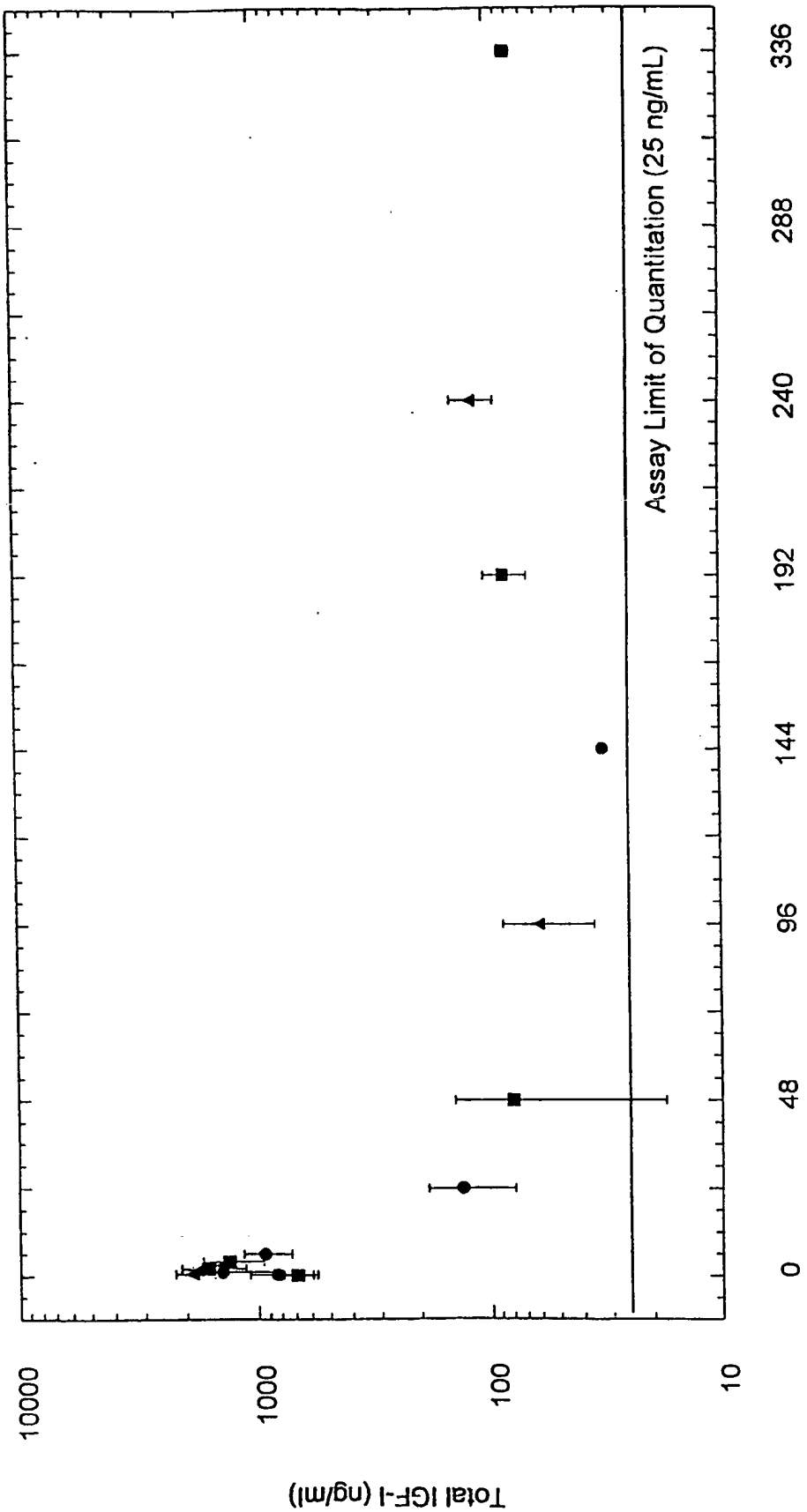

Total IGF-I (ng/ml)
10000
1000
100
10
0
48
96
144
192
240
288
336
Time (hr)
Assay Limit of Quantitation (25 ng/mL)

FIG. 3

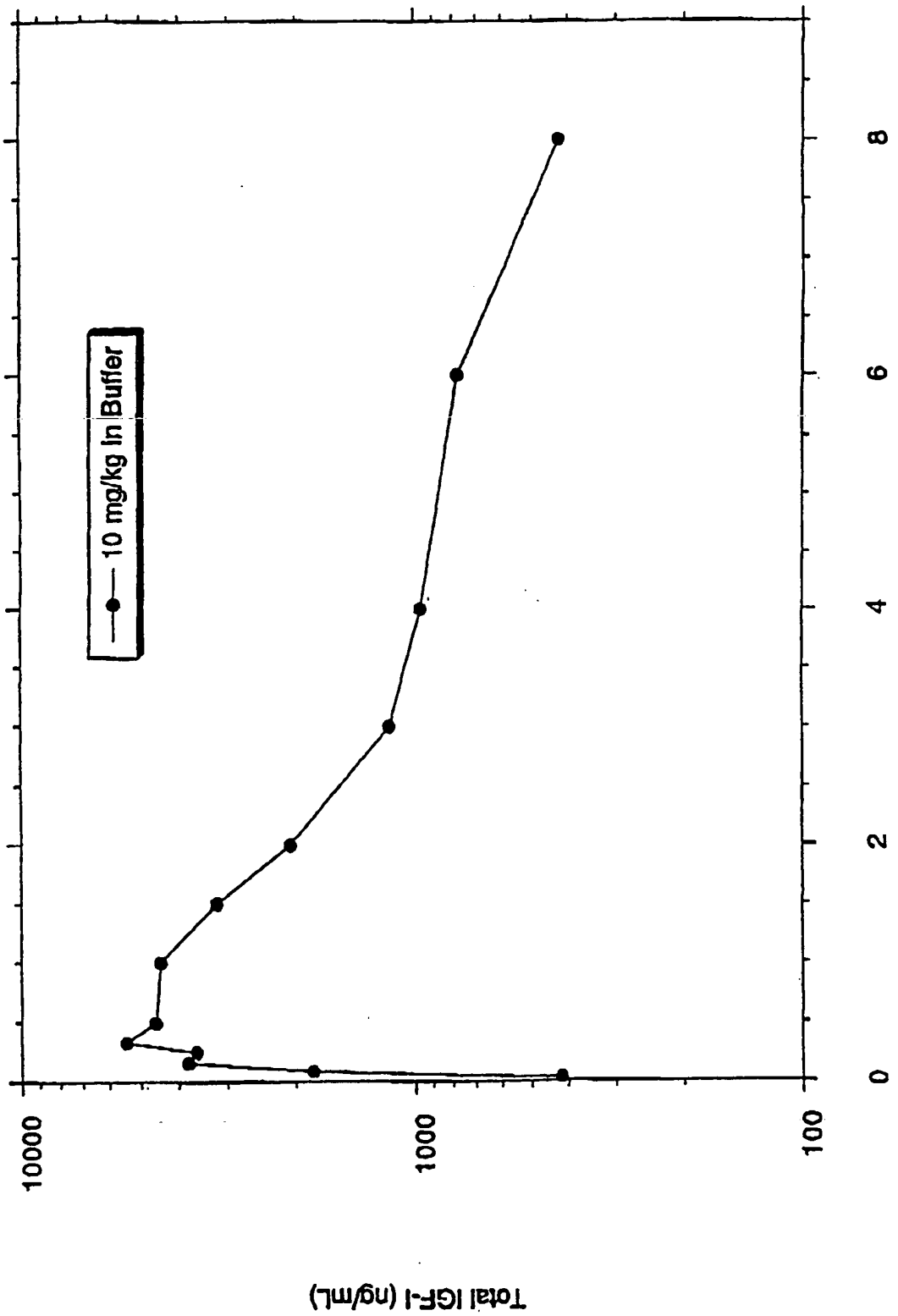

10 mg/kg In Buffer
Total IGF-I (ng/mL)
10000
1000
100
0
2
4
6
8
Time (hour)

FIG. 4

FIG. 5

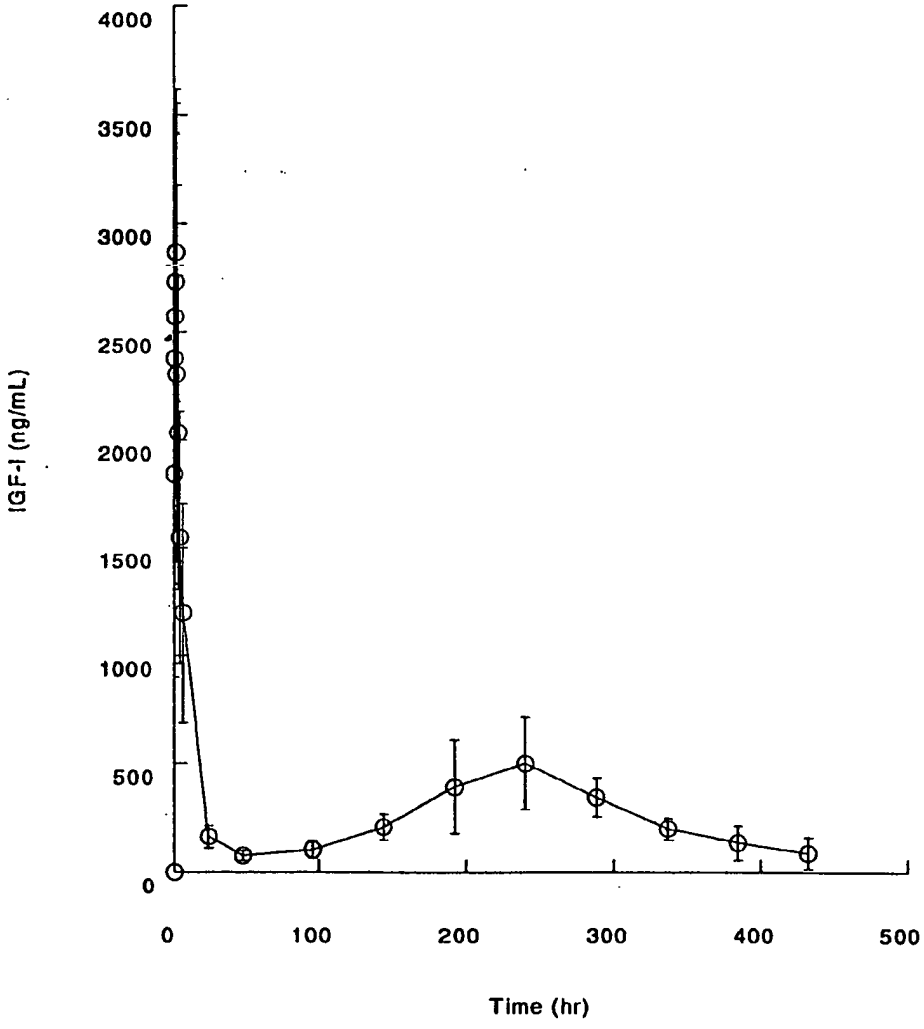
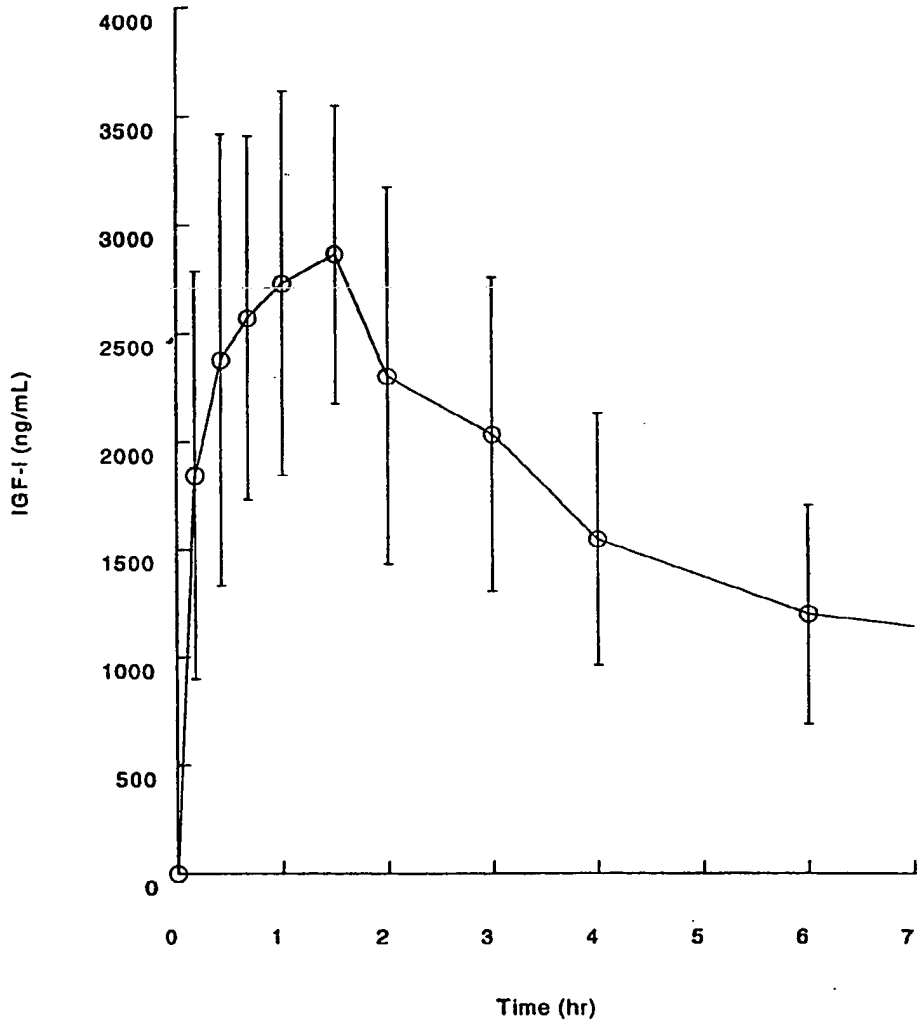

4000
3500
3000
2500
2000
1500
1000
500
0
IGF-I (ng/mL)
0
100
200
300
400
500
Time (hr)

FIG. 6


4000
3500
3000
2500
2000
1500
1000
500
0
IGF-I (ng/mL)
0
1
2
3
4
5
6
7
Time (hr)

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/US 98/23627

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/30 A61K9/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 11009 A (GENENTECH INC) 27 April 1995 see page 1, line 31 - page 2, line 9 see page 2, line 28-37 see page 4, line 19-23 see page 5, line 19-28 see page 6, line 17-33 see page 6, line 35 - page 7, line 12 see page 7, line 19-30 see claims 1,2,5,13,14 --- -/--	1,3,5-7, 10-14, 17-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 1999	13/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	La Gaetana, R

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/23672

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0440989 A	14-08-1991	AT 120763 T	15-04-1995
		CA 2033281 A	06-07-1991
		CA 2033535 A	06-07-1991
		DE 69018438 D	11-05-1995
		DE 69018438 T	24-08-1995
		DK 440989 T	01-05-1995
		ES 2070262 T	01-06-1995
		GR 3015712 T	31-07-1995
		IL 96839 A	30-03-1995
		JP 2099441 C	22-10-1996
		JP 4208228 A	29-07-1992
		JP 8018999 B	28-02-1996
		US 5210074 A	11-05-1993
EP 0297860 A	04-01-1989	AU 1998588 A	30-01-1989
		DD 271268 A	30-08-1989
		DE 3883645 D	07-10-1993
		DE 3883645 T	03-02-1994
		DK 675389 A	28-02-1990
		HU 209955 B	28-12-1994
		IE 65658 B	15-11-1995
		JP 2756131 B	25-05-1998
		JP 4502753 T	21-05-1992
		KR 131330 B	17-04-1998
		NO 301748 B	08-12-1997
		PH 26527 A	07-08-1992
		PT 87886 A,B	30-06-1989
		WO 8900049 A	12-01-1989
		US 5589169 A	31-12-1996

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr nal Application No
PCT/US 98/23627

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9511009 A	27-04-1995	AT 175110 T	15-01-1999
		AU 8017494 A	08-05-1995
		CA 2172508 A	27-04-1995
		DE 69415684 D	11-02-1999
		EP 0724433 A	07-08-1996
		JP 9504026 T	22-04-1997
EP 0442671 A	21-08-1991	AT 123413 T	15-06-1995
		CA 2036089 A	14-08-1991
		DE 69110150 D	13-07-1995
		DE 69110150 T	25-01-1996
		DK 442671 T	24-07-1995
		ES 2073117 T	01-08-1995
		FI 910674 A,B,	14-08-1991
		HK 188095 A	22-12-1995
		IE 68436 B	12-06-1996
		JP 2653255 B	17-09-1997
		JP 4321622 A	11-11-1992
		LT 441 A,B	25-11-1994
		LV 10041 A,B	10-05-1994
		LV 5754 A	20-12-1996
		NO 301405 B	27-10-1997
		PT 96727 A,B	31-10-1991
		RU 2098121 C	10-12-1997
		RU 2018306 C	30-08-1994
		US 5480656 A	02-01-1996
		US 5643607 A	01-07-1997
		US 5814342 A	29-09-1998

INTERNATIONAL SEARCH REPORT

Intern. al Application No
PCT/US 98/23627

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 442 671 A (TAKEDA CHEMICAL INDUSTRIES LTD) 21 August 1991 cited in the application see page 2, line 36-41 see page 3, line 11-12 see page 3, line 30-35 see page 3, line 40-41 see page 3, line 55 - page 4, line 13 see page 4, line 28 - page 5, line 10 see page 5, line 22-23 see claims 1,2,9,12-14	1,2,5,6, 11,12, 14,17, 19,20
A	JEFFERY H, DAVIS SS, O'HAGAN DT: "The preparation and characterization of poly(lactide-co-glycolide) microparticles.II. The entrapment of a model protein using a (water-in-oil)-in-water emulsion solvent evaporation technique" PHARMACEUTICAL RESEARCH, vol. 10, no. 3, 1993, pages 362-368, XP002097502 cited in the application see page 363, paragraph "Preparation of PLG microparticles with entrapped ovalbumin" see page 365, paragraph "Viscosity in the internal aqueous phase"	1,5,6, 10-12, 14,17-20

1